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Overexpression of *KAI1* Suppresses *in Vitro* Invasiveness and *in Vivo* Metastasis in Breast Cancer Cells[1]

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ABSTRACT

***KAI1* is a metastasis suppressor gene for human prostate cancer and is also involved in the progression of a variety of other human cancers. Previously, we have demonstrated that KAI1 expression was down-regulated in metastatic breast cancer cell lines as well as in highly aggressive breast cancer specimens. To determine whether KAI1 expression is responsible for the metastasis suppression in breast cancer, we transfected the human KAI1 cDNA into two highly malignant breast cancer cell lines, LCC6 and MDA-MB-231, which both have low levels of endogenous KAI1 expression. Parental, vector-only transfectants and KAI1 transfectant clones were injected into the mammary fat pads and tail veins, respectively, of athymic nude mice and assessed for both spontaneous and experimental lung metastasis. High KAI1 expression significantly suppressed the metastatic potential of KAI1-transfected LCC6 cells. Metastasis suppression correlated with the reduced rate of tumor growth and a decreased clonogenicity in soft agar. Furthermore, KAI1 expression significantly suppressed the *in vitro* cell invasion in KAI1-transfected MDA-MB-231 cells. Our results suggested that KAI1 may function as a negative regulator of breast cancer metastasis.**

INTRODUCTION

KAI1, a newly identified metastatic suppressor gene for prostate cancer, is located on human chromosome 11p11.2. KAI1 expression was reduced in human cell lines derived from metastatic prostate tumors as compared with its expression in normal prostate tissue (1). Down-regulation of the KAI1 protein was observed during the progression of human prostate cancer (2). Moreover, KAI1 was shown to suppress metastasis when introduced into rat AT6.1 prostate cancer cells (1). The role of KAI1 in tumor progression may not be limited to prostate cancer. KAI1 expression was reported to be correlated with the progression of a variety of human cancers such as non-small cell lung cancer, pancreatic cancer, bladder cancer, gastric cancer, and breast cancer (3–7). In addition, expression of KAI1 in colon cancer cells and melanoma cells resulted in reduced cell motility and invasiveness *in vitro* and in suppressed experimental metastasis *in vivo* in melanoma cells (8, 9). These findings, together with the fact that KAI1 protein has a nearly ubiquitous tissue distribution (1), suggested that *KAI1* may function as a metastasis suppressor gene in other types of cancers.

KAI1 is identical to CD82, which is a member of the TM4SF[4] (1). TM4SF members are characterized by four highly conserved transmembrane domains; two relatively divergent extracellular domains, the larger of which contains several conserved amino acid motifs; and two short cytoplasmic domains at the NH_2 and COOH termini. About 20 members of this family have been defined including MRP-1/CD9, TAPA-1/CD81, ME491/CD63, and KAI1/CD82. The precise biochemical function of the TM4SF is not clear yet; however, the current data suggest a role for this superfamily largely in the regulation of cell proliferation, activation, and motility (10, 11). A growing body of evidence suggests that CD9, one member in TM4SF, has at least a 54% identity with CD82 and is involved in cell motility and metastasis (12, 13). It was reported that the expression of CD9 was inversely correlated with metastasis in breast cancer (14). Expression of CD9 in malignant melanoma cells significantly suppressed the metastatic potential. Likewise, reduction or loss of ME491/CD63 expression was observed to be associated with increased metastatic ability of human malignant melanoma (15).

In our previous studies, we demonstrated that KAI1 expression, at both message and protein levels, was inversely correlated with the metastatic potential of some established human breast cancer cell lines. In addition, we also assessed KAI1 protein expression in human breast cancer specimens from patients with known clinical outcome, and we found that more malignant tumor types expressed significantly lower levels of KAI1 protein (16, 17). Our data suggested that KAI1 is down-regulated during the progression of human breast cancer. The aim of this study was to determine whether KAI1 could suppress invasive and metastatic ability of breast cancer cells. We transfected KAI1 full-length cDNA into highly malignant breast cancer cells, and we have measured cell proliferation, invasiveness, and *in vivo* metastasis. Our results demonstrated a significantly lower level of invasiveness and lung metastasis in KAI1 transfectants, which suggested that *KAI1* could function as a metastasis suppressor gene for human breast cancer.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

LCC6 and MDA-MB-231 are estrogen receptor-negative and progesterone receptor-negative breast cancer cell lines. LCC6 cell line is an ascites model of human breast cancer cell line MDA-MB-435 (18). Both cell lines were obtained from The Lombardi Cancer Center Tissue Culture Core Facility. They were cultured in modified improved MEM with Phenol Red containing 10% FBS. The neomycin-resistant KAI1 transfectants were maintained in the same medium but containing 600 μg/ml G418 for LCC6 transfectants and 1500 μg/ml G418 for MDA-MB-231 transfectants. All of the cell lines were free of *Mycoplasma* contamination.

Stable Gene Transfection

The pcDNA3-KAI1 vector was constructed by Dong *et al.* (1) and given to us as a generous gift. This vector was transfected into LCC6 and into MDA-MB-231 cells using the standard calcium phosphate method. Briefly, at day 1, the cells were plated at about 60–70% confluence in regular growing medium. At day 2, the medium was changed in the recipient cell cultures. DNA (10–20 μg) was mixed with calcium phosphate solutions, and this mixture was added dropwise to the cells. At day 3 (the day after transfection), the cells were washed and fed with fresh medium. At day 4, the antibiotics (G418) at appropriate concentrations were added to the cell culture, and the cells were grown for about 7–10 days before clone selection. pcDNA3 vector alone was also transfected into these cells to generate neotransfected control clones, designated LCC6/con and 231/con, respectively. G418-resistant clones overexpressing KAI1 were isolated by growth in selective medium.

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[4] The abbreviations used are: TM4SF, transmembrane 4 protein superfamily; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum.

Western Blot Analysis

Cell proteins were solubilized in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl, 1 mM EDTA, 1% Triton X-100, and 10% glycerol) containing proteinase inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 100 mM NaF, and 200 μM $NaVO_4$). Twenty μg of cell lysate were mixed with Laemmli's sample buffer without 2-mercaptoethanol, and boiled for 5 min. After SDS-PAGE (17.5%; Norvex, San Diego, CA), proteins were electrophoretically transferred to nitrocellulose (Amersham, Arlington Heights, IL) and probed with C33, a specific monoclonal antibody against KAI1 [a gift from Dr. Osamu Yoshie, Shionogi Institute for Medical Science, Osaka, Japan, (19)]. An enhanced chemiluminescence (ECL; Amersham) was used for signal detection.

FACS

Subconfluent cell monolayers were detached with 0.02% Na_2-EDTA in PBS. After two washes in cold PBS, 1×10^6 cells were incubated under gentle rotation with a specific anti-KAI1 antibody (Anti-CD82; Pharmagen, San Diego, CA) for 1 h at 4°C. This commercial antibody works very well for FACS and immunohistochemistry but not for Western analysis. After two washes in cold PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG for 30 min at 4°C. After washing, cells were suspended in PBS, and cell surface level of KAI1 protein was quantitatively measured by flow cytometry. Cells, stained with secondary antibody only, were measured at the same time to serve as background. The cell-surface-associated fluorescence was expressed as fold over background.

Anchorage-independent Growth Assay

A bottom layer of 0.1 ml improved MEM containing 0.6% agar and 10% FBS was prepared in 35-mm tissue culture dishes. After the bottom layer solidified, cells (1,000, 8,000, and 16,000 per dish, respectively) were added in a 0.8-ml top layer of 0.4% Bacto Agar and 5% FBS. All of the samples were prepared in triplicate. Cells were incubated for ~12 days at 37°C. Colonies larger than 60 μm were counted in a cell colony counter (Ommias 3600; Imaging Products International, Inc., Charley, VA).

Cell Invasion Assay

This assay was based on the principle of Boyden Chamber (20). Biocoat Matrigel invasion chambers were purchased from Becton Dickinson, and the protocol was provided by the manufacturer. Briefly, cells were plated in the top chamber (1.5×10^4 cells/chamber). An 8-μm pore size Matrigel-coated polycarbonate filter separated the top and bottom chambers. The bottom chamber contained 5% FBS as a chemoattractant. After 24-h incubation, the noninvasive cells were removed with a cotton swab. The cells that had migrated through the membrane and stuck to the lower surface of the membrane were fixed with methanol and stained with hematoxylin. For quantification, cells were counted under a microscope in five predetermined fields at ×200.

In Vivo Metastasis Assays

To measure spontaneous metastasis, 1×10^6 cells were injected into the subaxillary mammary fat pads at both sites of 4-to-6-week-old female athymic nude mice. Tumor sizes were monitored a week after inoculation of tumor cells. When the mean tumor diameter reached 1.0 cm, primary tumors were debulked. Mice were then maintained for an additional 1–2 months to allow further growth of lung metastases. To produce experimental lung metastasis, 1.5×10^7 cells were injected into the lateral tail veins of female athymic nude mice. After 4 weeks, the mice were necropsied, and the lungs were removed. Visible lung metastases were counted in Bouin's fixed tissues with the aid of a dissecting microscope.

Statistical Analysis

Anchorage-independent Growth Assay. A difference in colony-growth formation between control and KAI1 transfectants was tested using ANOVA (21). Cell type, control *versus* KAI1 transfectant, and numbers of cells plated (1000, 8000, or 16000) were included as predictor variables. Colony counts, the response variable, were transformed using the natural log of the count plus one to achieve an approximately normal distribution of the data.

Cell Invasion. Cell invasiveness was measured as the number of invaded cells per five fields. The comparison was made using ANOVA methods with a single contrast of 231/K2 and 231/K6 *versus* 231/con (21).

Tumor Growth. Tumor volumes were transformed by adding 1 and taking the natural log. The log tumor growth was presented graphically over time. A comparison was performed of the log-transformed tumor volumes of LCC/KH *versus* the control at day 26 using a nested ANOVA design (21) to account for the two-tumor observations per mouse. For reporting, means of the transformed were transformed back to the original units.

Metastasis. The number of lung metastases in the KAI1 transfectant LCC/KH was compared with control using the Mann-Whitney rank-sum test (22). Means and SEs, as well as the ranges for those mice with metastases, are reported for each.

Each test was considered significant if the P was less than 0.05. All of the analyses were performed using STATISTICA software (Statsoft, Inc., Tulsa, OK, 1998) or SigmaStat software (Jandel Scientific, San Rafael, CA).

RESULTS

Transfection of KAI1 cDNA and Selection of Stable Clones

To clarify whether KAI1 could suppress breast cancer invasion and metastasis, we transfected KAI1 full-length cDNA into breast cancer cell lines LCC6 and MDA-MB-231. LCC6 is an ascites model of the human breast cancer cell line MDA-MB-435. Compared with MDA-MB-435 cells, LCC6 cells grow tumors more rapidly *in vivo.* According to our *in vivo* metastasis assays, LCC6 cells develop *in vivo* metastases more rapidly and consistently than do MDA-MB-435 cells. MDA-MB-231 cells are reportedly less metastatic *in vivo* than are MDA-MB-435 cells but are much more invasive *in vitro* (23). Therefore, we chose LCC6 cells for *in vivo* metastasis assay and MDA-MB-231 cells for *in vitro* invasion assay. Both of these two cell lines have low levels of endogenous KAI1 expression according to our previous study (16). For both of these cell lines, only an expression vector was transfected as a negative control, and mixed populations of clones were selected to avoid clonal variation. These control cells were named as LCC/con and 231/con, respectively. About 20 single-cell clones were randomly selected, and the expression of KAI1 was confirmed by FACS assay, immunostaining, and Western blot. In LCC6 transfectants, one clonal population with a high level of KAI1 expression was selected by Western blot (data not shown). A FACS assay was then performed to quantify KAI1 expression level of this clonal population. Fig. 1 shows that KAI1 expression in this clone was significantly higher as compared with LCC/con and was designated as LCC/KH. In MDA-MB-231 transfectants, five clonal populations selected (231/K2, K5, K6, K11, and K16) had a similar level of KAI1 expression by Western blot (Fig. 2). Therefore, two of them (231/K2 and 231/K6) were arbitrarily selected for additional functional assays.

Effect of KAI1 on Anchorage-independent Cell Growth

The ability of LCC6 cells to form colonies in soft agar was examined because there exists a correlation between clonogenicity on soft agar and metastatic propensity (24). Fig. 3 shows that the clonal populations of KAI1 transfectants (LCC/KH) had significant reduction in their ability to form colonies in soft agar as compared with LCC/con cells ($P < 0.0001$).

Effect of KAI1 on Cell Invasion

An *in vitro* cell invasion assay was performed based on the principle of the Boyden chamber assay. The Matrigel matrix serves as a reconstituted basement membrane *in vitro*. The number of cells migrating through the Matrigel matrix was counted and the result is

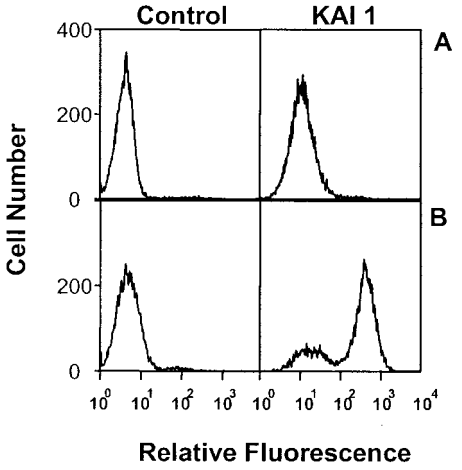

Fig. 1. Detection of KAI1 protein expression in transfectants of LCC6 cells by flow cytometry analysis. The cell surface levels of KAI1 protein in vector-transfected LCC6 cells (LCC/con; *A*), and KAI1-transfected LCC6 cells (LCC/KH; *B*) are shown. In the control panel (*Control*), cells were incubated without the primary antibody. In the KAI1 panel (*KAI 1*), anti-CD82 (PharMingen) was used as the primary antibody.

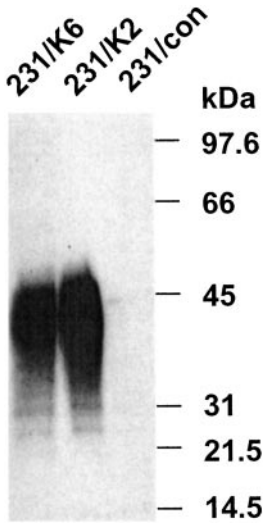

Fig. 2. Detection of KAI1 protein expression in transfectants of MDA-MB-231 cells by Western blot analysis. Proteins were extracted from subconfluent monolayer cell culture and separated by SDS-PAGE and then transferred to nitrocellulose membrane. The blot was probed with C33, a specific monoclonal antibody against KAI1 (a gift from Dr. Yoshie, Osaka). An enhanced chemiluminescence (ECL) was used for signal detection. 231/con are vector-transfected MDA-MB-231 cells. 231/K2 and 231/K6 cells are single clones of KAI1 transfectants showing similar levels of KAI1 expression. *kDa*, M_r in thousands.

presented in Fig. 4. Two clonal populations of KAI1-transfected MDA-MB-231 cells, 231/K2 and 231/K6, showed significantly reduced invasiveness as compared with 231/con cells ($P < 0.0001$). These data indicated that the enhanced expression of KAI1 in MDA-MB-231 cells is associated with reduced invasive capability.

Effect of KAI1 on *in Vivo* Tumor Growth and Metastasis

To unambiguously elucidate the suppressive effect of KAI1 on breast cancer metastasis, we injected LCC6 clones into athymic nude mice at mammary fat pads, and we monitored tumor growth as well as lung metastases.

Tumor growth. We began to measure tumor volumes 2 weeks after the injection and measured continuously every 3 days until primary tumors were debulked. Unlike *in vitro* cell growth, *in vivo* tumor growth rates of KAI1 transfectants appeared lower by the 3rd week of injection as compared with their control cells (Fig. 5). At day 26, the KAI1 transfectants had significantly lower tumor volume compared with the control ($P < 0.0001$). The means of the tumor volumes were 322.8 mm^3 and 33.5 mm^3 for LCC/con and LCC/KH, respectively.

Metastasis Step 1. Tumorigenic and metastatic properties of these clones are depicted in Table 1. All of the control cells (LCC6 wild type and LCC/con) formed tumors, whereas 27% (4 of 15) of mice inoculated with single clone of KAI1 transfectants (LCC/KH) did not grow tumors. These results suggest that KAI1 expression may have a suppressive effect on tumorigenicity. Also as shown in Table 1, LCC/KH had significantly fewer lung metastases than did control cells (represented by the mean number ± SE; $P = 0.017$).

Metastasis Step 2. To clarify whether the effect of KAI1 is directly targeted on cancer metastasis, experimental metastasis assay was performed using LCC/KH and control cells. Tumor cells were injected i.v. into the tail veins of nude mice, bypassing tumor formation at the primary sites. Table 2 illustrates that metastasis to lungs was suppressed by at least 50% in LCC/KH. These results demonstrated that the expression of KAI1 in LCC6 cells significantly suppressed metastatic ability in athymic nude mice.

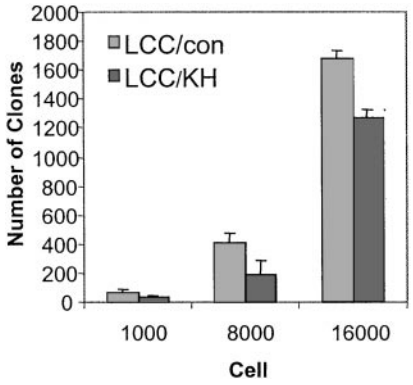

Fig. 3. Clonogenicity of LCC6 clones in soft agar. Cells were incubated in soft agar for 12 days at 37°C. Colonies larger than 60 μm were counted in a cell colony counter. Data represent the mean ± SE of triplicate dishes. LCC/KH had significantly reduced ability of colony formation as compared with LCC/con ($P < 0.0001$).

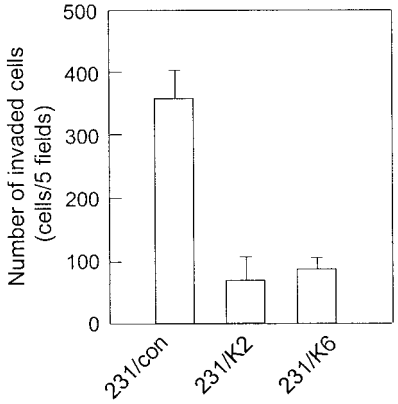

Fig. 4. Effect of KAI1 expression on *in vitro* invasiveness of breast cancer cells. MDA-MB-231 clones were plated in invasion chambers with filters coated with reconstituted basement membrane. After overnight incubation, cells that had migrated through the filters were stained with hematoxylin and counted in five randomly selected fields under a microscope at ×200. The bars represent the mean ± SE of three chambers from two separate experiments. KAI1 transfectants 231/K2 and 231/K6 had significantly reduced invasive ability compared with 231/con cells ($P < 0.0001$).

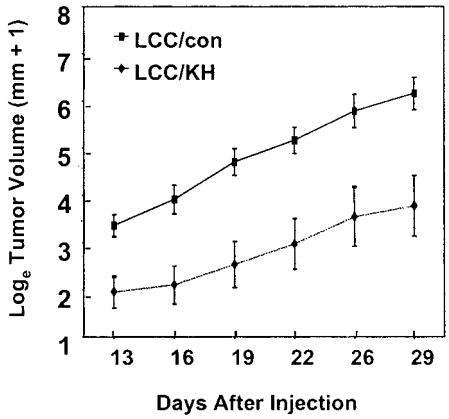

Fig. 5. Effect of KAI1 expression on *in vivo* tumor growth. LCC6 clones (1×10^6/per site) were injected into the subaxillary mammary fat pads at both sites of female nude mice ($n = 11$ for LCC/con and LCC/KH). Tumor volumes were measured every 3 days continuously from the 2nd week to the 4th week after the injection. The data were transformed by adding 1 and taking the natural log. Each value represents the mean ± SE from a total of 22 transformed tumor measurements from 11 mice (2 tumors each) for each cell type. LCC/KH transfectants displayed decreased tumor size compared with the control cells.

DISCUSSION

In our previous studies, we observed that KAI1 expression, at both message and protein levels, was inversely correlated with the metastatic potential of some established breast cancer cells. In addition, KAI1 was found to be down-regulated in the majority of aggressive breast tumors examined (16, 17). To further elucidate the effect of KAI1 on breast cancer metastasis, we transfected KAI1 cDNA into two highly malignant breast cancer cell lines, LCC6 and MDA-MB-231, which both have low levels of endogenous KAI1 expression (16). LCC6 was derived from MDA-MB-435, a commonly selected cell model for studying breast cancer metastasis. According to our preliminary experiments, LCC6 cells developed *in vivo* metastases more rapidly and consistently than their parental MDA-MB-435 cells. Therefore, we chose LCC6 cells for most of our functional assays. On the basis of published data and results from our own experiments, MDA-MB-231 is more invasive *in vitro* than is MDA-MB-435, despite being less metastatic *in vivo*. Consequently, we chose MDA-MB-231 cells for cell migration and invasion assays.

In agreement with studies of prostate cancer, colon cancer and melanoma cells by Dong *et al.* and Takaoka *et al.* (1, 8, 9), our data also indicate that KAI1 expression did not alter *in vitro* cell proliferation under either regular or serum-deprived conditions (data not shown). However, in contrast to their results, our *in vivo* studies revealed that there appeared a decrease in tumor volumes of KAI1 transfectants 3 weeks after inoculation (Fig. 5). Tumor size was not measured beyond this date because primary tumors were debulked to allow mice a longer survival to develop more visible lung metastases. Surprisingly, we observed that KAI1 expression appeared to have an inverse correlation with tumorigenicity. This is an interesting finding because no previous reports supported the role of KAI1 in primary tumor formation. Consistent with this finding, the ability of colony formation in soft agar of KAI1 transfectants was also significantly suppressed. Our results suggest that KAI1 may be a negative regulator of primary tumor growth in breast cancer. However, at this step, we cannot draw any definite conclusion because the measurements of tumor size were not able to be continued beyond 1 month. One may argue that the growth rates of these cells could be the same but only with the late onset of tumor formation. In addition, we had only one single clone with high KAI1 expression; thus, the result is preliminary. More studies using clones with different KAI1 expression levels are necessary to further elucidate this point.

KAI1 has been extensively studied for its involvement in the progression of different human cancers. However, limited information has been available in terms of its suppression of *in vivo* metastasis; even less is known about its molecular mechanism as a negative regulator of cancer progression. We correlated KAI1 expression with the ability of transfected cells to form lung metastases using both spontaneous and experimental metastasis assays. Our results demonstrated clearly that KAI1 expression in breast cancer cells significantly suppressed the metastatic potential in the KAI1 highest-expressing clone. These results are different from the study of Phillips *et al.* (25) in which the transfection of KAI1 cDNA into MDA-MB-435 cells resulted in clones that did not have a significantly decreased *in vivo* incidence of lung metastases. In their study, they noted that average number of metastases per lung varied over a large range. However, they tested only four mice for each clone. Therefore, it may not be valid to draw the conclusion with such a small sample size and a big variation. In our xenograft assay with LCC6 cells, we had about 15 mice per group. In Phillips' study, it was shown that the primary tumors and the metastatic lesions of the transfectants had decreased levels of KAI1 protein compared with the inoculated cells. Therefore, the possibility that KAI1 levels dropped below a threshold level required for metastasis suppression could not be eliminated. We constantly checked the KAI1 expression levels in KAI1-expressing clone before each experiment to make sure the KAI1 expression was

Table 2 *Metastasis analysis of KAI1-transfected LCC6 cells (i.v. injection)*

LCC clones (1×10^7/mice) were injected i.v. into the tail veins of female nude mice. Lung metastases were quantified 5 weeks after the injection. A significant reduction ($P = 0.024$) was observed in the number of lung metastases by LCC/KH cells as compared with LCC/con cells by Mann-Whitney rank-sum test. LCC6 is included as a reference, but was not included in the statistical analysis.

Cell line	Mean no. of metastases ± SE
LCC6	107.6 ± 36.4
LCC/con	139.8 ± 40.6
LCC/KH	47.6 ± 23.6

Table 1 *Metastasis analysis of KAI1-transfected LCC6 cells (mammary fat pad injection)*[a]

	Tumor	Metastases			
Cell line	Incidence	Mean counts ± SE	Incidence/mice with primary tumor	Non-zero counts range	P[b]
LCC6[c]	13/13	23.85 ± 8.84	11/13	3–105	N/A
LCC/con	15/15	18.53 ± 5.42	13/15	1–69	N/A
LCC/KH	11/15	4.73 ± 2.37	10/11[d]	2–35	0.017[e]

[a] LCC clones (1×10^6/per site) were injected into the subaxillary mammary fat pads at both sites of female nude mice. Primary tumors were debulked when the mean tumor diameters reached ~10 mm. About 2–3 months after the initial innoculation, mice were killed, and lung metastases were counted under a dissecting microscope.
[b] The p refers to a rank test of metastases count comparing LCC/KH with LCC/con (control).
[c] LCC6 is included as a reference only. It was not included in any analyses.
[d] These numbers assume that only mice with tumors produced metastases.
[e] Significant at the 0.05 level.

not lost. To verify the effect of KAI1 on breast cancer metastasis, we further performed the experimental metastasis assay. By directly injecting cells into tail veins of nude mice, the tumor formation at the primary sites was bypassed. There are two advantages associated with this assay. First of all, we could determine whether the effect of KAI1 is directly on metastasis or indirectly through the interference with primary tumor formation. Secondly, it takes a much shorter time to finish this experiment as compared with the spontaneous metastasis assay (1 month *versus* 3 months). Therefore, it is less likely that *KAI1* gene is inactivated during the experiment. Consistently with the result from spontaneous metastasis assay, experimental metastasis was suppressed by KAI1 overexpression. In addition, the effect of KAI1 appeared to be directly targeted on metastasis and, KAI expression was not, at least not completely, inactivated during the experiment.

It has to be noticed that high KAI1 expression level did not eradicate lung metastases. This implies that KAI1 is not the sole factor responsible in suppressing breast cancer metastasis. *KISS-1*, a metastasis suppressor gene for melanoma, was shown to suppress *in vivo* metastasis when transfected into a breast cancer cell line, MDA-MB-435. Like KAI1 transfectants, *KISS-1* transfectants also did not result in 100% suppression of lung metastases (26). Therefore, it is highly likely that multiple genes are required for the complete suppression of breast cancer metastasis.

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